New and Notable

Star Light, Star Bright, First Molecule I See Tonight

Christopher M. Yip*

Institute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto, Ontario, Canada

With apologies for taking poetic license with a favorite nursery rhyme, the drive for higher temporal and spatial resolution optical imaging, coupled with novel fluorophore designs, has resulted in spectacular new insights into the spatial and temporal nature of cellular and molecular dynamics, interactions, and structures. However, it is arguably true that these insights and interpretations rely heavily on a priori understanding (or perception) of the stability, reliability, and robustness of the imaging optics and in particular a complete understanding of the optical characteristics of both the excitation and emission paths.

Owing to its exceptional axial resolution, total internal reflection fluorescence microscopy (TIRF) has long been the platform of choice for investigating cellular and molecular dynamics in the cell-substrate regions (1). It has, for example, enabled remarkable insights into the mechanisms of secretory vesicle transport, exocytosis, and focal adhesion structure and dynamics. Whether prism-based, or, as has become more commonplace, objective-based using high numerical-aperture (NA) objectives, critical to the success of TIRF imaging has been the ability to reproducibly and accurately ensure that the incident light impinges on the substrate-sample interface at the appropriate illumination angle, as defined by Snell's law. As a staple platform for live cell imaging, TIRF microscopy has benefited greatly from continual advances in optics, software, and fluorescent probes. Indeed, it has been the integration of these tools and techniques that has fueled rapid advances in superresolution localization microscopies, such as FIONA, (F)PALM, and STORM (2,3).

While the founding principle of TIRF microscopy—that of creating a surface evanescent wave—is relatively simple in concept, it has long been recognized that numerous technical challenges exist that need to be carefully considered and addressed to provide truly quantitative insights. These challenges, especially in the context of cell imaging, include understanding how the properties of the evanescent wave (both in the axial direction and across the field of view) could be affected by the sample itself. This is particularly critical because the overarching premise in TIRF is that the axial penetration depth is well described and that the illumination, and thus accessible sampling volume, is strictly defined. Moreover, there is often a tacit assumption that the observed fluorescence is entirely a consequence of the expected near-field excitation. This, however, is not necessarily the case, and one needs to be aware of the potential convolving effects of far-field excitation on the TIRF images and any subsequent quantitative interpretation.

In a set of companion articles in this issue of *Biophysical Journal*, Brunstein et al. (4,5) describe their recent efforts to identify and then address these key challenges. Their motivation was the following: In laser-based objective TIRF systems, the evanescent wave generated through a high-NA objective yields an anisotropic illumination profile across the field of view, an effect that can be obviated through beam scanning (6). This strategy has been shown to be an attractive and relatively readily implemented approach for generating an azimuthally symmetric illumination field (7,8). Indeed, in their first article, Brunstein et al. (4) clearly illustrated the benefits of beam-scanning TIRF, revealing how dramatic differences in feature intensity can arise simply as a consequence of the position of the excitation beam in the objective's back focal plane. Failing to account for this imaging artifact could, as the authors clearly describe, result in misinterpretation as to the basis for the intensity differences, especially in the context of subcellular structures. Although beam scanning or what they describe as "spinning" has helped to address the issue of in-plane illumination field symmetry, it does not easily answer the question of penetration depth, or that of axial uniformity.

As alluded to earlier, a key advantage of TIRF is that the excitation volume, as described axially by the exponentially decaying evanescent field, is effectively restricted. One tends to tacitly assume that the observed fluorescence thus arises from structures located within this volume. However, what has become clear is that far-field excitation can, and does, occur, primarily through scattering of the evanescent wave. What is less clear is the origin of the scattering—is it sample-specific? Or is it due to the instrument optics? Or some combination thereof? Using negative staining imaging, the authors were able to confirm that, not unexpectedly, cells themselves can act as scattering sources. What was remarkable was that this effect was not strongly correlated with incident angle, suggesting that sample scattering was not the primary contributor to the observed far-field excitation. This was made even more convincing because the authors chose to use two different cell types in their studies—weakly scattering astrocytes, and strongly scattering BON cells. What the authors were able to show, by positioning an objective above the high-NA TIRF objective and collecting the scattered far-field

Submitted January 2, 2014, and accepted for publication January 10, 2014.

*Correspondence: christopher.yip@utoronto.ca

Editor: David Piston.

0006-3495/14/03/0987/2 $2.00

http://dx.doi.org/10.1016/j.bpj.2014.01.009

excitation light, was that it was the high-NA objective, or perhaps more strictly, the excitation path optics, that formed the leading cause of the far-field excitation light.

These results are certainly illuminating because they illustrate two now-critical requirements for quantitative TIRF imaging:

1. Isotropic illumination, as can be achieved using beam-spinning TIRF; and
2. Compensation for far-field excitation that, in large part, is due to the internal optics of the microscope itself.

Addressing this second requirement is the thrust of the second article in the companion set by Brunstein et al. (5), wherein the authors propose an innovative strategy that marries TIRF illumination with supercritical-angle fluorescence (SAF) detection. This coupled approach addresses the issue of far-field excitation in TIRF due to scattering and instrument optics by effectively only detecting emission from fluorophores located in the near-field. Indeed, this very concept of exploiting the advantages of near-field excitation and emission was recently proposed in a compelling review by Axelrod (9). To accomplish this using high-NA objectives, and building off work by Barroca et al. (10), the authors implemented a dual-view imaging strategy that allows them to simultaneously acquire both a full-field image and a slightly restricted field-of-view image comprising the undercritical angle fluorescence (UAF) image (10). Because the full-field image contains contributions from both the UAF and SAF, subtracting the UAF component yields a high spatial resolution SAF, or virtual SAF, image. By collecting beam-spinning TIRF images as a function of incident angle (variable-angle TIRF) and implementing the virtual SAF image processing approach, it now becomes possible to perform accurate optical sectioning and obtain truly quantitative TIRF data.

This series of companion articles provides an articulate and cogent exposition of the challenges in quantitative live cell imaging now emerging in TIRF and superresolution imaging, challenges that are in large part a consequence of technical advances in high-NA objectives but also inherent to the biological systems themselves. By cleverly integrating a number of powerful imaging modalities—dark-field scattering, SAF, variable-angle TIRF, beam-spinning TIRF—along with innovative technical approaches for rapid image acquisition and processing into a flexible and open architecture microscope, Brunstein et al. (4) have now provided the community with a roadmap for the ready implementation of these platforms into existing commercial systems. With that in mind, it bears mentioning that the ease with which they were in fact able to develop these solutions can be attributed to their use of an optical bench-based TIRF microscope. Herein, of course, lies what will likely be a major challenge for the general practitioner of TIRF or localization microscopies—can these approaches be implemented into commercial platforms? While beam scanning has already made it into a number of commercial TIRF systems, this only serves to help create a uniform illumination field and remove directionality. There now exists an opportunity for developers to create a turnkey platform that enables virtual SAF detection. Provided such a platform can be developed, there will surely be rapid adoption by the community of the powerful combination of beam-spinning TIRF and virtual SAF detection for quantitative live cell superresolution imaging.

REFERENCES

1. Axelrod, D. 2008. Chapter 7: total internal reflection fluorescence microscopy. *Methods Cell Biol.* 89:169–221.
2. Hoffman, M. T., J. Sheung, and P. R. Selvin. 2011. Fluorescence imaging with one nanometer accuracy: in vitro and in vivo studies of molecular motors. *Methods Mol. Biol.* 778:33–56.
3. Sengupta, P., S. van Engelenburg, and J. Lippincott-Schwartz. 2012. Visualizing cell structure and function with point-localization superresolution imaging. *Dev. Cell.* 23:1092–1102.
4. Brunstein, M., M. Teremetz, …, M. Oheim. 2014. Eliminating unwanted far-field excitation in objective-type TIRF. Part I. Azimuthal beam spinning is not a cure-all. *Biophys. J.* 106:1020–1032.
5. Brunstein, M., K. Herault, and M. Oheim. 2014. Eliminating unwanted far-field excitation in objective-type TIRF. Part II. Combined evanescent-wave excitation and supercritical-angle fluorescence detection improves optical sectioning. *Biophys. J.* 106:1044–1056.
6. van 't Hoff, M., V. de Sars, and M. Oheim. 2008. A programmable light engine for quantitative single molecule TIRF and HILO imaging. *Opt. Express.* 16:18495–18504.
7. Mattheyses, A. L., K. Shaw, and D. Axelrod. 2006. Effective elimination of laser interference fringing in fluorescence microscopy by spinning azimuthal incidence angle. *Microsc. Res. Tech.* 69:642–647.
8. Fiolka, R., Y. Belyaev, …, A. Stemmer. 2008. Even illumination in total internal reflection fluorescence microscopy using laser light. *Microsc. Res. Tech.* 71:45–50.
9. Axelrod, D. 2013. Evanescent excitation and emission in fluorescence microscopy. *Biophys. J.* 104:1401–1409.
10. Barroca, T., K. Balaa, …, E. Fort. 2012. Full-field near-field optical microscope for cell imaging. *Phys. Rev. Lett.* 108:218101.